DEVELOPMENTAL MODIFICATIONS IN THE SAND DOLLAR CAUSED BY COBALTOUS CHLORIDE IN COMBINATION WITH SODIUM SELENITE AND ZINC CHLORIDE[1]

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Studies on the effects of chemical agents on early developmental patterns in echinoderms have shown that similar modifications may often be caused by entirely different agents. Some groups of totally different substances will cause entodermization of the young embryo while others will produce the opposite effect (ectodermization) if concentrations and exposure periods are carefully controlled. Some will increase the area of the ventral field while others will decrease it. In recent studies (Rulon, 1952, 1953, 1955, 1956) it has been found that compounds such as sodium selenite, nickelous chloride, zinc chloride, and cobaltous chloride will all cause the loss of bilaterality and a polar elongation of the larva. Such larvae differentiate with reference to the new pattern and are very similar to each other, irrespective of the compound used.

When two unlike substances produce similar effects certain questions may be asked: (1) Do these agents affect identical reaction systems in the morphogenetic process? Or (2) are *different* loci in the reaction complex affected in such a way that the final results are the same? A definite answer to either question is difficult to make but it is believed that at least a partial answer may be suggested by the use of combinations of the different agents.

In our experiments it has been customary to submit the eggs and early embryos to a wide range of concentrations when testing the effects of any particular agent. This range usually extends from a concentration that is lethal in a few hours to one that has little or no effect. The range is commonly set up so that the succeeding steps are each one-half the concentration of the preceding (*i.e.*, $M/100$, $M/200$, $M/400$, $M/800$, $M/1{,}600$, $M/3{,}200$ . . .). When newly fertilized eggs from the same lot are distributed throughout the various concentrations, interesting comparisons may often be made. The stronger solutions commonly give proportionate inhibition—that is, all structures are grossly inhibited. With decreased concentrations the inhibition may be disproportionate (differential) in that certain processes or structures are strongly affected while others are affected slightly if at all. The differential inhibition of one structure often provides for the physiological release of another to the extent that the structure not inhibited increases in size beyond the normal.

The present work deals chiefly with combinations of agents. Since previous investigations have shown that cobaltous chloride, sodium selenite, and zinc chloride

[1] This investigation was supported by the Graduate School of Northwestern University. The writer is also much indebted to Dr. L. R. Blinks, Director of the Hopkins Marine Station, Pacific Grove, California, for many considerations.

all cause the development of sand dollar embryos that are quite similar, it seemed important to determine if these agents could replace one another in inhibitory solutions. Would the effects of these different agents be antagonistic, additive, or possibly synergistic?

Material and Methods

This work was conducted at the Hopkins Marine Station, Pacific Grove, California, during the summers of 1954–55. The adult sand dollars (*Dendraster excentricus*) were dredged from Monterey Bay and maintained in the laboratory in running sea water. Ovaries were exposed by removing the oral surfaces of the animals. The bright red eggs, exuding in droplets, were washed into finger bowls. After several washings in sea water the ova were fertilized by the addition of a few drops of sperm suspension. Only ova that were over 95 per cent fertilizable were used. All test solutions were made up in sea water and the controls and tests were always from the same batch of eggs. All eggs developed under uncrowded conditions in finger bowls, out of direct sunlight and under the moist conditions of the aquarium room where the temperature varied by no more than one degree from 18 degrees C. and smoking was not permitted.

Experimental

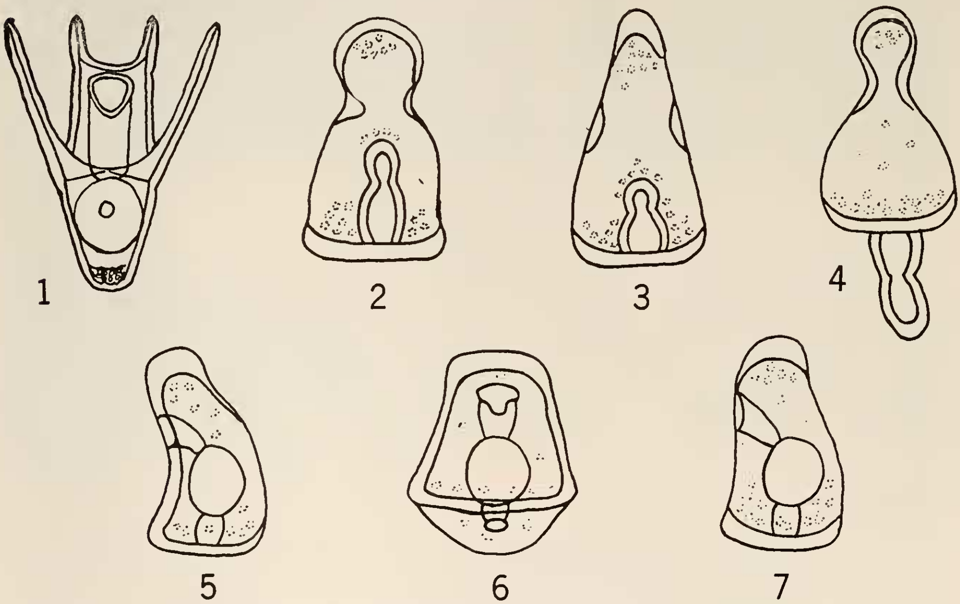
1. *Continuous exposure of newly fertilized eggs to single and combination solutions of cobaltous chloride and sodium selenite.* It would be repetitious to report here all of the modifications caused by solutions of only cobalt and selenium (see Rulon, 1952, 1956). The object of this paper is to make comparisons between the effects of solutions containing the ions singly and in combinations at critical concentrations of the range. Accordingly, only the effects of the following solutions will be discussed although additional data have been obtained from various other concentrations and combinations:

Solution 1—Sea water control
2—$M/800$ cobaltous chloride
3—$M/1{,}600$ cobaltous chloride
4—$M/800$ sodium selenite
5—$M/1{,}600$ sodium selenite
6—$M/1{,}600$ cobaltous chloride—$M/1{,}600$ sodium selenite (50 cc. of $M/800$ $CoCl_2 \cdot 6H_2O$ plus 50 cc. of $M/800$ Na_2SeO_3)
7—$M/3{,}200$ cobaltous chloride—$M/3{,}200$ sodium selenite (50 cc. of $M/1{,}600$ $CoCl_2 \cdot 6H_2O$ plus 50 cc. of $M/1{,}600$ Na_2SeO_3)

After 48 hours almost all (98 per cent) in solution 1 (control) had developed into normal free-swimming bilateral plutei with well-differentiated oral and anal arms and with full skeletal development (Fig. 1). In solution 2 all were radial in symmetry and almost all (95 per cent) showed polar elongation (Figs. 2–4). Most showed differentiation of an apical lobe (Figs. 2, 4). All had thickened basal regions and commonly a basal circle of cilia was to be seen. Exogastrulation was present in 20–30 per cent and skeletal development was inhibited in all. All were slow-moving bottom forms. In solution 3, which was one-half the strength in cobaltous chloride of solution 2, less than 10 per cent resembled the radial elongated

forms of solution 2. Instead, approximately 90 per cent were bilateral, free-swimming larvae (as Figs. 5–7). The majority of these larvae showed well-differentiated entera and stomodaea but no differentiation of skeleton or arms. There were some indications of polar elongation but they were not nearly so pronounced as in the larvae that had developed in solution 2. Approximately 20 per cent showed exogastrulation.

After 48 hours in solution 4 almost all (95 per cent) were radial forms with very little movement (Figs. 8–11). Over 75 per cent showed considerable polar elongation (Figs. 10–11) with differentiation of apical and basal lobes. No skeleton differentiated and many had basal ciliated bands. Only a few exogastrulae appeared. Approximately 75 per cent of the eggs developing in solution 5 resulted in bilateral

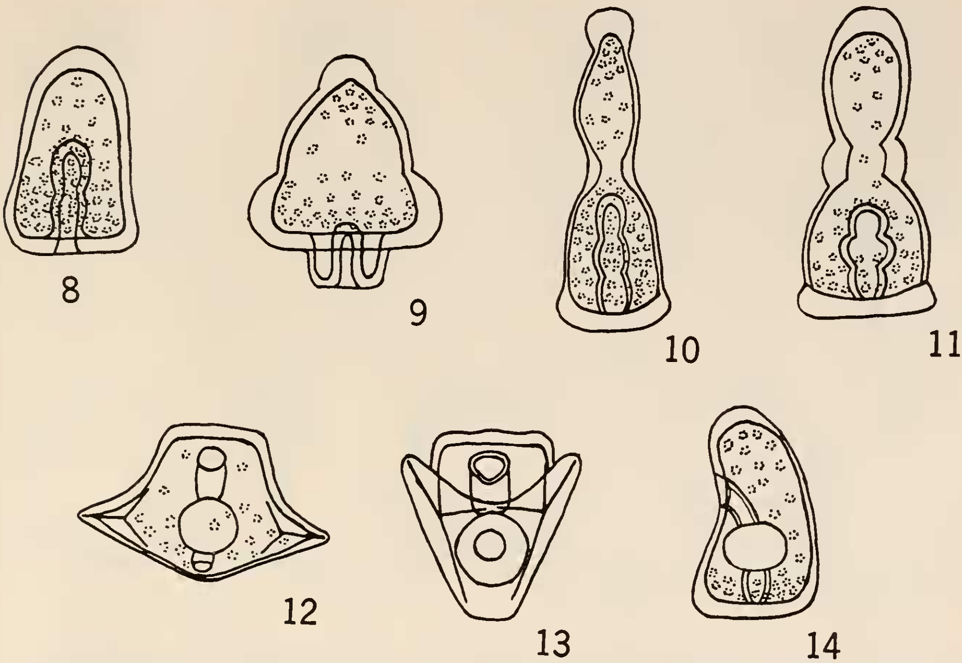


FIGURES 1–7. Figure 1, normal 48-hour larva. Figures 2–4, 48-hour larvae that have been exposed continuously to $M/800$ cobaltous chloride. Figures 5–7, 48-hour larvae exposed continuously to $M/1{,}600$ cobaltous chloride.

plutei with short anal arms containing skeleton and at increased angles (Figs. 12–13). Oral lobes were broad but poorly differentiated. The remainder of the larvae in solution 5 graded from slightly bilateral forms without skeleton (Fig. 14) into the polar elongated radial larvae of higher concentrations (Figs. 10–11). Only an occasional exogastrula was seen.

Almost 100 per cent of the eggs developing in solution 6 became radial or near radial larvae (Figs. 15–18). Of these, over 50 per cent showed polar elongation with oral and basal lobes (Figs. 15–17). Approximately 10 per cent were exogastrulae. In solution 7, there was only an occasional elongated radial form while approximately 90 per cent were bilateral free-swimming larvae with ventral ciliated bands but no skeleton or arms (Figs. 19–21). Approximately 10 per cent were exogastrulae but the remainder had well-differentiated entera with stomodaea.

From these experiments it was shown that when the eggs of *D. excentricus* were exposed to a combined solution of cobalt and selenite ($M/1,600\ CoCl_2$–$M/1,600\ Na_2SeO_3$) they differentiated according to a pattern of radial symmetry very much as if they had been exposed to single solutions of double strength of either agent. That is, while $M/1,600$ cobalt alone caused 10 per cent radial and $M/1,600$ selenite alone caused 25 per cent radial, together they caused 100 per cent radial (approximately 100 per cent radial forms are caused by $M/800$ of either agent). In the case of polar elongation the combined solution gave a considerably higher percentage than did the single solutions ($M/1,600\ CoCl_2$ or $M/1,600\ Na_2SeO_3$) but not as high as single solutions of double strength. Exogastrulation was highest in the cobalt,



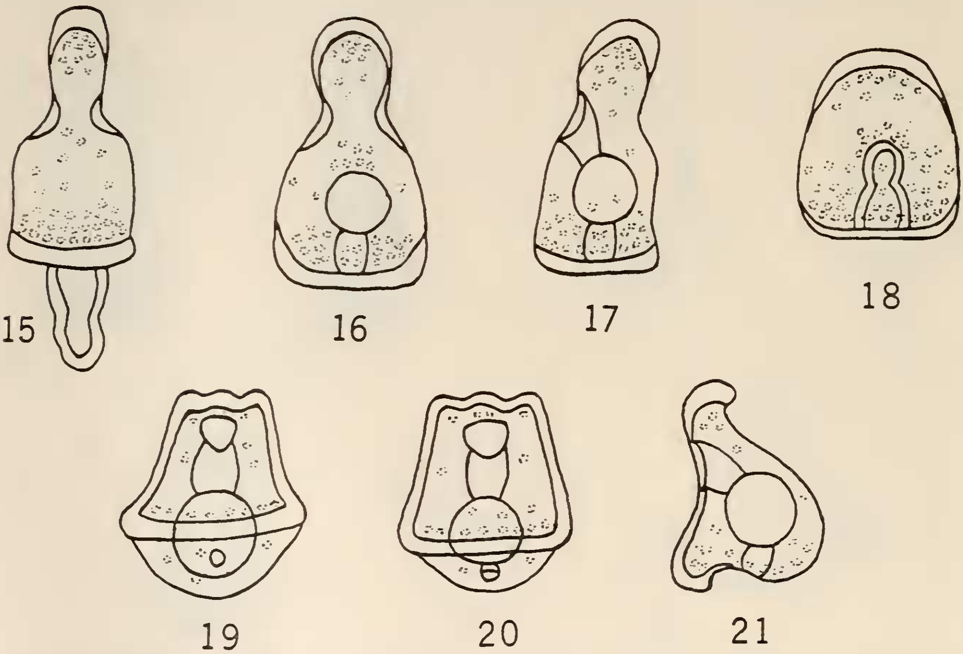
FIGURES 8–14. Figures 8–11, 48-hour larvae that have been exposed continuously to $M/800$ sodium selenite. Figures 12–14, 48-hour larvae that have been exposed continuously to $M/1,600$ sodium selenite.

next in the combination, and least in the selenite solutions. The data have demonstrated that the actions of these two different agents are additive in most respects in affecting developmental pattern but that there are also some effects caused by one agent but not (at least to an appreciable degree) by the other at the concentration used.

2. *Continuous exposure of newly fertilized eggs to single and combination solutions of cobaltous chloride and zinc chloride.* As in the preceding experiments, eggs from the same lot were placed in wide ranges (single and combination) of concentrations. Since the single effects have previously been reported (Rulon, 1955, 1956) only the effects of selected solutions on development shall be reported here. The solutions were as follows:

Solution 8—Sea water control
9—$M/3,200$ cobaltous chloride
10—$M/80,000$ zinc chloride
11—$M/160,000$ zinc chloride
12—$M/3,200$ cobaltous chloride—$M/160,000$ zinc chloride (50 cc. of $M/1,600$ $CoCl_2 \cdot 6H_2O$ plus 50 cc. of $M/80,000$ $ZnCl_2$)

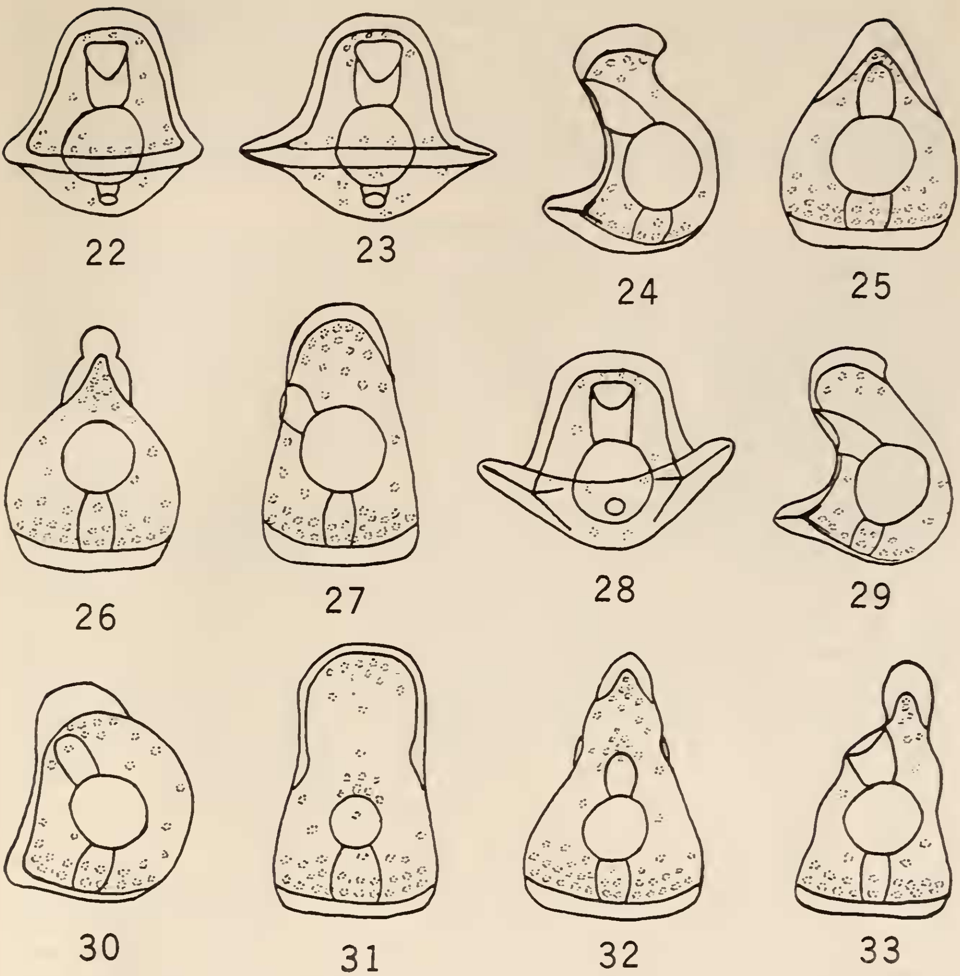
After 48 hours the eggs developing in the sea water control (solution 8) were practically 100 per cent normal free-swimming bilateral plutei with good development of oral and anal arms. In solution 9 all were slightly flattened bilateral larvae



FIGURES 15–21. Figures 15–18, 48-hour larvae that have been exposed continuously to a combination solution ($M/1,600$ cobaltous chloride-$M/1,600$ sodium selenite). Figures 19–21, 48-hour larvae that have been exposed continuously to a combination solution ($M/3,200$ cobaltous chloride-$M/3,200$ sodium selenite).

which were either actively swimming or moving about near the bottom of the culture (Figs. 22–24). Approximately 20 per cent had short anal arms with skeletal spicules and 10–20 per cent had undergone exogastrulation. While these larvae were definitely in advance of those treated with cobalt solutions of twice the strength (solution 3), they still showed markedly the effects of the agent.

In solution 10 all of the larvae (Figs. 25–27) were slow-moving and radial with a large differentiated gut which commonly extended to the apical end (Fig. 25) although there were approximately 10 per cent which showed exogastrulation. These larvae had no skeleton and there was an excess of internal cells. Most showed apical thickenings or extensions although the polar elongation fell short of



FIGURES 22–33. Figures 22–24, 48-hour larvae that have been exposed continuously to *M*/3,200 cobaltous chloride. Figures 25–27, 48-hour larvae that have been exposed continuously to *M*/80,000 zinc chloride. Figures 28–30, 48-hour larvae that have been exposed continuously to *M*/160,000 zinc chloride. Figures 31–33, 48-hour larvae that have been exposed continuously to a combination solution (*M*/3,200 cobaltous chloride-*M*/160,000 zinc chloride).

that noted in certain effective solutions of cobalt and selenium. In solution 11 (which was one-half the concentration of zinc chloride as solution 10) over 90 per cent of the larvae were bilateral (Figs. 28–30) and over 50 per cent of these had short anal arms with skeleton. The remaining 10 per cent approached radial symmetry and there was approximately 5 per cent exogastrulation.

In solution 12 (combination solution) over 90 per cent were elongated radial forms with neither skeleton nor arms (Figs. 31–33). Slight bilaterality was evidenced in the remaining 10 per cent. Most had well-developed entera but 20–30 per cent had undergone exogastrulation.

These experiments show that zinc chloride causes modifications similar to those caused by cobaltous chloride but in concentrations that are 1/100th those of the latter. The addition of $M/3{,}200$ cobalt to $M/160{,}000$ zinc gives an effect which is approximately that of a single solution of zinc of twice the concentration ($M/80{,}000$) but not that of a single solution of cobalt of twice the strength ($M/1{,}600$). Instead the effect is that of a cobalt solution of four times the concentration ($M/800$).

Discussion

Previous work by the author has shown that several substances are effective in causing polar elongation and differentiation around a radial symmetry in the developing sand dollar embryo. The present data show that cobalt and selenite at the same concentrations will produce such modifications. They also show that the effects of these two different agents were additive in causing radial symmetry and almost additive in polar elongation and exogastrulation.

Other work has indicated that the radial symmetry described here is the result of a process of ventralization (see Rulon, 1949) rather than a direct inhibition of the factors which give bilaterality to the normal embryo. It has been shown (see Child, 1941) that the ventral side of the early blastula has greater indophenol oxidase (cytochrome oxidase) activity than the dorsal side, even though visible morphological differences are not apparent. Neither cobalt nor selenite appears to inhibit the activity of ventral as much as dorsal regions. The ventral area therefore spreads until it encircles the entire embryo. It is suggested that the similar effects of cobalt and selenite may be related to their known inhibitory action of thiol groups (see Rulon, 1955) and that enzymes important in symmetry relationships bear active sulfhydryl radicals. It is further suggested that these enzymes are more dorsally located at the stage of development preceding visible bilaterality. Cytochrome oxidase, whose greater activity at the ventral side has been proven, does not possess an active sulfhydryl radical (Sumner and Somers, 1953, p. 9).

Polar elongation seems to have certain factors in common with ventralization. Child (1941) also showed a polar gradient of cytochrome oxidase activity in these eggs with the highest activity at the animal pole. If it is assumed that cobalt and selenite are inhibiting activities other than cytochrome oxidase and more basally located, then it would follow that the apical end would grow and become extended at the expense of basal regions.

Zinc was highly effective in causing ventralization and polar elongation, and at a concentration far below that of the other two agents. In other words, it was more effective in inactivating, or partially inactivating, certain of the factors or processes concerned with bilaterality and polarity. It may not be unreasonable to suggest that the affinity of the thiol groups of certain enzymes for this ion may be much greater than for cobalt or selenite ions. However, the picture may be more complicated, as shown by the fact that a solution of zinc ($M/160{,}000$) which is effective in causing 10 per cent radial forms will, when administered along with $M/3{,}200$ cobalt, which causes no radial forms, cause 90 per cent radials. This would seem to suggest a synergistic action although the complexity of the phenomenon does not lend itself to an easy interpretation.

Cobalt was much more effective in the concentration used than was selenite in causing exogastrulation and skeletal inhibition. This may be because the differ-

entiation of gut and skeleton is of a more specific nature and therefore subject to more specific influences than is either ventralization or polar elongation.

Summary

1. Newly fertilized eggs of *D. excentricus* were allowed to develop in single and combined sea water solutions of cobaltous chloride, sodium selenite, and zinc chloride.

2. Combination solutions of cobalt and selenite were additive in causing the development of radial larvae and almost additive in causing polar elongation of the larvae.

3. In solutions of equivalent strength the development of exogastrulae was highest in cobalt, next in the combination, and least in the selenite solutions.

4. Solutions of zinc caused effects that were similar to those of cobalt but in concentrations that were 1/100th the latter.

5. Combination solutions of cobalt and zinc gave effects that indicated synergistic action.

6. It is suggested that the effects of cobalt, selenite, and zinc are through their reaction with the thiol groups of certain enzymes and that the greater effect of zinc is because of a greater affinity for such groupings.

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